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***Mycobacterium tuberculosis* RD-1 Secreted Antigens as Protective and Risk Factors for Tuberculosis**

Niladri Ganguly^{1,2} and Pawan Sharma¹

¹*International Centre for Genetic Engineering and Biotechnology,
Aruna Asaf Ali Marg,
New Delhi,*

²*Wellcome Trust Biocentre, College of Life Sciences,
University of Dundee, Dundee,*

¹*India*

²*UK*

1. Introduction

Mycobacterium tuberculosis (Mtb) infects about 8 million people every year and causes death of about 2-3 million (Raviglione, 2003). In recent times, there has been a wider spread of tuberculosis, mainly due to emergence of multi drug resistance (MDR) bacilli and enhanced susceptibility to the disease by patients infected with human immunodeficiency virus (HIV) (Elliot et al., 1995; Chintu and Mwinga, 1999). Transmission of the infection by Mtb bacilli is air borne and occurs through inhalation of aerosol containing the bacilli exhaled by coughing, sneezing or spitting by patients suffering from pulmonary tuberculosis. The inhaled bacilli are engulfed by the alveolar macrophages, where the bacilli are able to persist successfully in a latent or proliferating state. This persistence is achieved by modulation of several intracellular signaling pathways in order to create a suitable environment for the bacilli. The interplay of mycobacteria with host signaling pathways is a complex and dynamic process that is not clearly understood. Mtb secretes several molecules that modulate the signaling pathways (Koul et al., 2004). Most of these molecules commonly target macrophages, which helps the bacilli to evade innate immune response and propagate throughout the system (Rosenberger and Finlay, 2003).

The proteins secreted by Mtb have gained attention in recent years as putative vaccine and diagnostic candidates (Harboe et al., 1996; Colangeli et al., 2000). But there have been recent reports about their role in modulation of macrophage signaling pathways leading to compromise of macrophage functions (Trajkovic et al., 2002; Pym et al., 2003; Guinn et al., 2004). Thus the secretory proteins can act as risk or virulent factors too. This notion is also supported by the fact that only live but not dead bacilli can down regulate macrophage functions (Malik et al., 2001). In this chapter, we focus our discussion on the role of the

proteins secreted by region of difference-1 (RD-1), the region that is deleted in all the strains of *M. bovis* BCG.

2. Genetic architecture of Region of Difference-1 (RD-1)

Comparative genome analysis using DNA microarray, bacterial artificial chromosomes (BAC) and the subtractive hybridization between virulent and attenuated strains of *Mtb* complex and *M. bovis* BCG identified several regions of difference (RD) (Behr et al., 1999; Gordon et al., 1999; Mahairas et al., 1996). A gene segment of 9.5kb that encompasses nine open reading frames (ORF) of Rv3871-Rv3879c is present in virulent strains of *Mtb*, and which is deleted consistently in all the strains of *M. bovis* BCG (Cole et al., 1998). This region was designated as RD-1. Two of these ORFs, Rv3874 and Rv3875, encode 10-kDa culture filtrate protein (CFP-10) and 6-kDa early secreted antigenic target (ESAT-6) protein respectively. Interestingly, deletion of the RD-1 fragments from *Mtb* causes loss of its virulence, while introduction of the RD-1 locus into *M. bovis* BCG or *M. microti* resulted in increased virulence and survival properties (Behr, 2002; Pym et al., 2002; Lewis et al., 2003; Demangel et al., 2005). This review will focus on the role of these two proteins in modulation of the macrophage signaling pathways and macrophage functions for the bacteria to persist for longer time. We also discuss about the potential role of these proteins as vaccine candidates owing to their high immunogenicity.

3. Structural biology of CFP-10 and ESAT-6

The ORFs Rv3874 and Rv3875 encoding CFP10 and ESAT6 respectively are cotranscribed into a single RNA product (Cole et al., 1998). Nuclear magnetic resonance (NMR) spectroscopy showed that CFP10 exhibits very little secondary structure and consists mostly of random coils, which are unstructured. The ESAT6, on the other hand has 75% secondary structure in the form of α -helices (Renshaw et al., 2002). One interesting study showed that CFP10 and ESAT6 forms a tight 1:1 complex where both proteins adopt more stable and folded configuration than the native moieties (Renshaw et al., 2002; Lightbody et al., 2004). The complex formation between the two proteins is hydrophobic in nature and led to a significant increase in the helical content of the two proteins. Inside the core of the complex, helix-turn-helix motifs of the two proteins form a quad-helix bundle (Renshaw et al., 2005). Within the complex, the flexible C-terminus of CFP-10 is involved in binding to the cell surface. This was confirmed by the fact that deletion 87 amino acids at C-terminus of CFP-10 inhibited the binding of complex to the cell surface, while deletion of the same in ESAT-6 had no effect (Renshaw et al., 2005). The complex formation between the two proteins was reversible and the complex broke down to individual proteins at 53.4°C. The complex was also shown to be more stable to proteolytic digestion by trypsin (Meher et al., 2006). The enhanced stability to proteolytic digestion caused lower T cell activation compared to ESAT-6 alone (Marei et al., 2005). ESAT-6 protein was also found to have auto-proteolytic activity; it can self cleave-off six amino acids at the C-terminus which are responsible for its binding to the cell surface. A mutant ESAT-6 lacking these six amino acids was unable to bind to cell surface (Pathak et al., 2007).

4. Use of CFP10 and ESAT6 as tools for diagnosis and vaccine

CFP-10 and ESAT-6 were identified in a screen to identify the proteins present in culture filtrates of *Mtb* and *M. bovis* BCG, which could induce T cell mediated response. (Andersen et al., 1991a; Andersen et al., 1991b; Andersen et al., 1994; Weldingh et al., 1998; Weldingh et al., 1999). The screen yielded six low molecular weight antigens *viz.* Rv3871, Rv3872, Rv3873, CFP-10, ESAT-6 and Rv3878. These antigens when expressed and purified as recombinant proteins gave strong humoral response in tuberculous guinea pigs while only two antigens i.e. CFP-10 and ESAT-6 showed strong delayed type hypersensitivity (DTH) reaction in the guinea pigs (Weldingh et al., 1999). CFP10 and ESAT6 are potent T cell antigens and induce strong T cell response. In mice infected with *Mtb*, CFP-10 specific T cells were observed at quite early stage of infection in lungs. These T cells were activated by CFP-10 epitopes and were recruited in large numbers (Kamath et al., 2004). This resulted in production of large amounts of interferon- γ (IFN- γ). Recombinant CFP-10 has also been shown to be a potent T cell antigen, inducing T cell proliferation and IFN- γ production in peripheral blood mononuclear cells in about 70% of purified protein derivative (PPD) positive asymptomatic individuals. CFP-10 was also shown to induce delayed type hypersensitivity (DTH) in *Mtb* infected guinea pigs but not in *M. bovis* BCG infected guinea pigs (Colangeli et al., 2000). ESAT-6 is also a RD-1 antigen inducing robust levels of IFN- γ by T cells in early stages of *M. tuberculosis* infection (Porsa et al., 2006; Ravn et al., 1999; Skjot et al., 2000; de Jong et al., 2006). Two different T cell epitopes were observed in mice, which were recognized by different MHCII molecules under different circumstances (Dietrich et al., 2005).

5. Macrophage subversion by CFP10 and ESAT6

Despite their well-known role as T cell antigens, CFP10 and ESAT6 modulate several pathways inside the macrophage, thereby creating a suitable environment to persist inside the host cell. Studies from our lab have shown that CFP-10 and ESAT-6 downregulates the production of reactive oxygen species (ROS) inside the macrophages; which in turn dampens the NF- κ B transactivation property (Ganguly et al., 2008a, Ganguly et al., 2008b). The inhibition of ROS production was greater with the CFP10:ESAT6 complex compared to the individual proteins. Most of the effects of these proteins seem to be mediated by Toll-like receptors (TLR). Analysis of global phosphoproteome in CFP-10 treated J774.1 macrophages showed that CFP-10 caused de-phosphorylation of a large number of macrophage proteins (Basu et al., 2006; Basu et al., 2009). The de-phosphorylation occurs due to increase in activity of membrane tyrosine phosphatases SHP-1 and SHP-2 (Src homology domain proteins). The increased phosphatase activity is due to reduction in production of ROS inside the macrophages. The ROS production in macrophages occurs through NADPH oxidase pathway. These observations suggest that upon binding of CFP-10 and ESAT-6 to macrophage surface, *Mtb* is able to reduce the burst of ROS inside the cell which contributes to bactericidal activity. Thus it might be one of the survival strategies of the bacilli. *Mtb* contains several enzymes to deal with the ROS/oxidative burst like catalase, peroxidase (Kat) (Sherman et

al., 1995; Manca et al., 1999; Ng et al., 2004) as well as superoxide dismutases Sod A and Sod C (Piddington et al., 2001; Zhang et al., 1991). ESAT-6 was also found to inhibit mitogen activated kinase/extracellular signal regulated kinases 1/2 (MAPK/ERK1/2). This occurs due to some phosphatase activity in the nucleus which dephosphorylates ERK1/2. This resulted in reduction in lipopolysaccharide (LPS) induced expression of transcription factor c-myc (Ganguly et al., 2007). ESAT-6 also reduced the LPS-induced expression of several genes like *IL-1 β* , *Bax*, *Icam-1* and *tnfr-1*. Recent studies have shown that ESAT-6 binds to toll-like receptor-2 (TLR2) on the macrophage surface; and the six amino acids at the C-terminus of the protein are critical for its TLR2 binding (Pathak et al., 2007). This binding caused inactivation of transcription factors interferon regulatory factors (IRF) and NF- κ B. Recent observations from our lab have shown that ESAT-6 down-regulates IFN- γ inducible expression of type I and type IV isomers of MHC class II transactivator (CIITA) in macrophages (Kumar et al., 2011). Interestingly, the downregulation of type I CIITA was independent of TLR-2 while the effect on type IV CIITA was mediated through TLR-2. This suggests that ESAT-6 may bind to other TLRs or some other receptor on macrophages. Another study has shown that ESAT-6 was able to induce apoptosis in human monocytic cell line THP-1 through activation of caspases (Derrick et al., 2007). It was also shown that ESAT-6 could induce pore formation on the surface of some cell types.

Apart from macrophages, CFP-10 and ESAT-6 also modulate functions in dendritic cells (Natarajan et al., 2011; Trajkovic et al., 2004). Studies have shown that CFP-10 induced differentiation of bone marrow cells into dendritic cells (DC) and this involved activation of NF- κ B (Latchumanan et al., 2002). CFP-10 also induced maturation of DCs, which caused downregulation of pro-inflammatory cytokines like interleukin-2 (IL-2) and IFN- γ (Natarajan et al., 2003). The CFP-10-differentiated and CFP-10-matured DCs when cultured with the Mtb whole-cell-extract primed T cells, showed reduced levels of pro-inflammatory cytokines IL-12p40 and IFN- γ along with elevated levels of anti-inflammatory cytokines IL-10 and transforming growth factor β (TGF- β) (Balkhi et al., 2004). Therefore CFP-10 primes DCs to have reduced efficacy to eliminate Mtb. CFP-10 also reduced ROS production during differentiation of DCs compared to the positive stimulator granulocyte macrophage colony-stimulating factor (GM-CSF). This downregulation of ROS resulted in increased survival of *M. bovis* BCG in these DCs (Sinha et al., 2006). The CFP-10 differentiated DCs also had reduced levels of chemokines RANTES and IP-10 upon infection by mycobacteria (Salam et al., 2008).

6. Virulence linked to CFP10/ESAT6 secretion

CFP-10 and ESAT-6 proteins are secreted without any secretory signal sequence outside the host cell. The secretory apparatus that helps in the secretion of these two proteins have been characterized recently (Guinn et al., 2004; Fortune et al., 2005; Brodin et al., 2006). The CFP10/ESAT6 secretion system is an active process involving several genes, some of them being ATP hydrolyzing chaperones (Stanley et al., 2003; Converse and Cox, 2005; Stanley et al., 2007). Mutations in these genes caused significant reduction in virulence of Mtb in mouse model (Stanley et al., 2003; Majlessi et al., 2005). This

attenuation could be due to the inhibition of CFP10/ESAT6 secretion, which further highlights the role of these two proteins in modulation of macrophage function. A study has shown that one of the components of ESX-1 secretion system, Rv3871 binds to the C-terminus of CFP-10, and this facilitates the secretion of both CFP-10 and ESAT-6. Mutations at the C-terminus resulted in loss of binding to CFP-10 and impaired secretion of the two proteins (Champion et al., 2006). This suggests that CFP-10 and ESAT-6 might be secreted in the form of heterodimeric 1:1 complex out of the cell. The ESX-1 system has four paralogues in Mtb and some of them have been shown to be essential for in-vitro growth of the bacilli (Simeone et al., 2009). In the ESX-1 system, Rv3868, Rv3869, Rv3870, Rv3871 and Rv3877 have been shown to be essential for CFP-10/ESAT-6 secretion while loss of Rv3865 and partial loss of Rv3866 did not affect protein secretion, rather it caused attenuation of the bacilli (Brodin et al., 2006). Thus Rv3865/3866 might be some virulence factor that does not control ESAT-6 secretion. Studies with mutant bacilli showed that ESX-1 system is required for the induction of type I IFN induction that in turn contributes to the spread of the bacilli (Stanley et al., 2007). In *Mycobacterium marinum*, the CFP-10/ESAT-6 secretion manipulates the phagosome-lysosome fusion. Mutations in this secretion system results in enhanced phagosome-lysosome fusion and reduced survival of mycobacteria (Tan et al., 2006; Majlessi et al., 2005; Champion et al., 2006; Xu et al., 2007; Lee et al., 2001). Analyses of deletion mutants of ESAT-6 have identified the key amino acids in complex formation, virulence and secretion (Brodin et al., 2005). The Trp-X-Gly motif on ESAT-6 is involved in complex formation with CFP-10, virulence and induction of specific T cell responses whereas mutations in the six amino acids at the C-terminus had no effect on secretion but caused attenuation. At acidic pH (as normally found in phagosomes), ESAT-6 dissociated from its complexing partner CFP-10 and bound to liposomes, which caused lysis of the liposomes (de Jonge et al., 2007). This could be a mechanism for the Mtb to escape degradation within the phagosome. In dendritic cells, Mtb translocated from phagolysosome to cytoplasm, which is dependent upon the CFP-10/ESAT-6 secretion (van der Wel et al., 2007). This translocation resulted in the death of the host cell.

7. Conclusions

The interaction between mycobacteria and the host macrophage or dendritic cells is very complex and dependent on multiple factors. In this review, we have focused mainly on the modulating activities of CFP10/ESAT6, the molecules which are being evaluated as vaccine candidates, indicating that they may act like double-edged sword generating a favorable response from the host immune system. Apart from CFP-10/ESAT-6, several other protein antigens are also being reported which modulate the macrophage response to Mtb. Further studies are undergoing in our lab to elucidate the finer mechanisms by which these proteins function.

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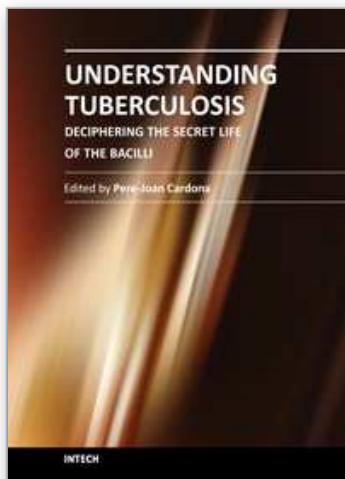
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Mycobacterium tuberculosis, as recent investigations demonstrate, has a complex signaling expression, which allows its close interaction with the environment and one of its most renowned properties: the ability to persist for long periods of time under a non-replicative status. Although this skill is well characterized in other bacteria, the intrinsically very slow growth rate of Mycobium tuberculosis, together with a very thick and complex cell wall, makes this pathogen specially adapted to the stress that could be generated by the host against them. In this book, different aspects of these properties are displayed by specialists in the field.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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